Final Technical Report

Date of Final Report: December 31, 2006
EPA Grant Number: RD827354C005
Center Name: University of Rochester–EPA PM Center
Center Director: Gunter Oberdorster
Title: Ultrafine Particle Cell Interactions: Molecular Mechanisms Leading to Altered Gene Expression
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Institutions: University of Rochester
EPA Project Officer: Stacey Katz/Gail Robarge
Project Period: June 1, 1999–May 31, 2005 (no-cost extension to May 31, 2006)
Period Covered by the Report: June 1, 1999–May 31, 2006
RFA: Airborne Particulate Matter (PM) Centers (1999)
Research Category: Particulate Matter

Objective(s) of the Research Project:

Hypothesis

Numerous epidemiological studies have found a correlation between exposure to respirable airborne particulate matter (PM) and increased mortality and adverse respiratory health effects, including the development of emphysema, chronic bronchitis, and asthma and acute and chronic cardiovascular effects. On the tissue and cellular level, PM-deposition insults can result in pulmonary inflammation, airway hyperreactivity, epithelial cell damage, and increased epithelial permeability. A key biological effect of inhaled PM has been the recognition of cardiac and cardiovascular effects. The mechanism of effects of inhaled PM on the cardiovascular system was more difficult to discern until recent data indicated the transport of ultrafine PM across the pulmonary epithelium into the vascular bed (Oberdorster, et al., 2002; Kreyling, 2002; Oberdorster and Utell, 2002). Thus, the possibility of direct particle cell interaction with the vascular endothelium becomes a distinct mechanistic possibility.

The formation of reactive oxygen species (ROS) and subsequent lipid peroxidation is believed to play a major role in toxicity; however, the rate of formation of ROS can depend on synergistic effects between components of PM and on the presence of relatively benign materials. The direct mechanisms by which the wide variety of airborne PM types impact target cells in the respiratory and cardiovascular system is diverse, thus severely complicating schemes to monitor the potential impact of the release of such particulates into the atmosphere.

The experiments performed within this project were designed to address specific mechanistic hypotheses regarding the interactions between inhaled ultrafine particles and specific pulmonary and cardiovascular cell populations. The proposed *in vitro* experiments were intended to provide a link between the whole animal and controlled clinical (human) exposures, described in the other programs of this PM Center, by elucidating specific mechanisms that are triggered following particle cell contact and to test the specific hypothesis that many of the subsequent

physiologic effects are the consequences of cellular oxidative stress, cell activation, and apoptosis.

A key component of the studies included an expansion of the effect of PM on pulmonary cells to cells of the cardiovascular system and to assess both particle-cell and cell-cell interactions on the processes of cell activation and alteration of gene expression. Work by a number of authors has suggested that production of both inflammatory and fibrotic mediators following particle interaction is not limited to classic inflammatory cells, but that pulmonary parenchymal elements including epithelial cells (type II, Clara cells) and fibroblasts may also contribute to the milieu.

Summary of Findings: A key component of our studies was to examine particle cell interactions in individual cell populations to begin to assess the role of ultrafine particles (UFP) in altering inflammatory gene expression by an oxidant-related mechanism. In our experiments, in collaboration with Core 4, we were able to define susceptible populations on the basis of age as well as prior or concurrent infection.

To test the hypothesis that increased susceptibility of aged animals is due to cell intrinsic differences in oxidant sensitivity, we evaluated the effect of age on the response of cells to particles. We compared macrophage production of cytokines following lipopolysaccharide (LPS) and particles from 22-27 month old rats to cells from 10-12 week old rats. Baseline (unstimulated) production of MIP-2 (and TNF) was elevated 30-50% in these cells as well as increased response to exogenous stimulus. Increased production of prostaglandin E_2 (PGE_2) by alveolar macrophages from "aged" animals, an endpoint chosen to better correlate with the animal studies (Core 4) and the human clinical studies (Core 3) was observed when cultured in the presence of LPS used as a positive particle control, and LPS plus particles confirming age effects for a number of endpoints.

An important development during this project was the ability to use laboratory generated ultrafine particles containing various metals. The choice of the specific metal was based on the data provided by our Chemical UFP characterization (Core 1) that iron is among the most abundant metal constituents. This material was produced by our particle generation Core. We compared macrophage production of cytokines following LPS and particles (with C/Fe) incubation with cells from 20-22 month old and 8-10 week old mice. Baseline MIP-2 and TNF was significantly elevated in cells from "old" mice. After stimulation, the old mice were also found to be more responsive.

When particles and LPS were combined as a stimulus an enhanced effect is observed only in the "old" cells except at the highest dose of particles. Most significant, in the context of our investigation of age effects and the ability of particles to induce effects at low dose, was the fact that in the aged animals co-administration of particles and LPS leads to synergistic effects at the lowest dose of particles. This result is somewhat similar to results obtained in the *in vivo* studies in which enhanced response to combined insult was noted in aged rats.

One marker that has proven useful in assessing cellular response to PM is the production of prostaglandins (PGs). By measuring changes in PGs, we could indirectly monitor activity of COX-2, the rate limiting enzyme and also determine the role of PGs in pulmonary and systemic

inflammation. Stimulation of young and old cells with a combination of ultrafine C/Fe particles and LPS lead to an increase in PGE_2 production (Figure 1). As with MIP-2 (and TNF) this was mainly observed in the cells from the old mice. This is consistent with our other age experiments and reinforces the hypothesis that age is related to increased PM susceptibility.

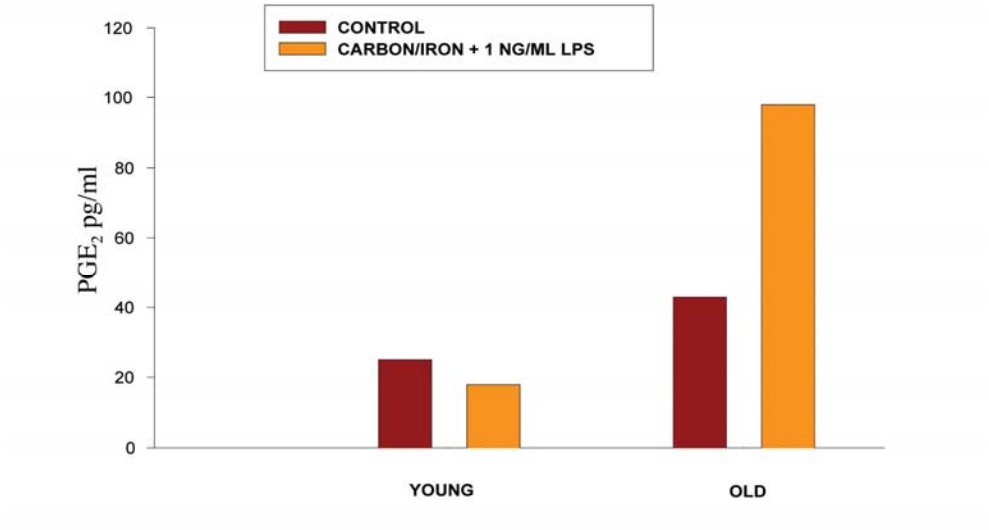

Figure 1.

We also developed reagents and approaches that would allow extension of our *in vitro* studies to human cells while also developing a test of our oxidant stress hypothesis. We developed a human lung cell line, A549, which was stably transfected with a reporter gene that in other studies has been shown to be responsive to oxidant stress. Using this transfected A549 cell line, we were able to detect changes in gene expression at particle doses below 1 $\mu g/cm^2$ (Figure 2). This clearly puts us in the realistic range of ultrafine PM mass burdens. In future studies, together with our particle generation Facility Core we will determine if this relationship will be maintained for particles of different composition or with ambient particles collected using the Harvard ultrafine particle concentrator that we have available for our use. Our initial studies comparing cytokine analysis with luciferase activity show a reasonable correlation between these two measurements.

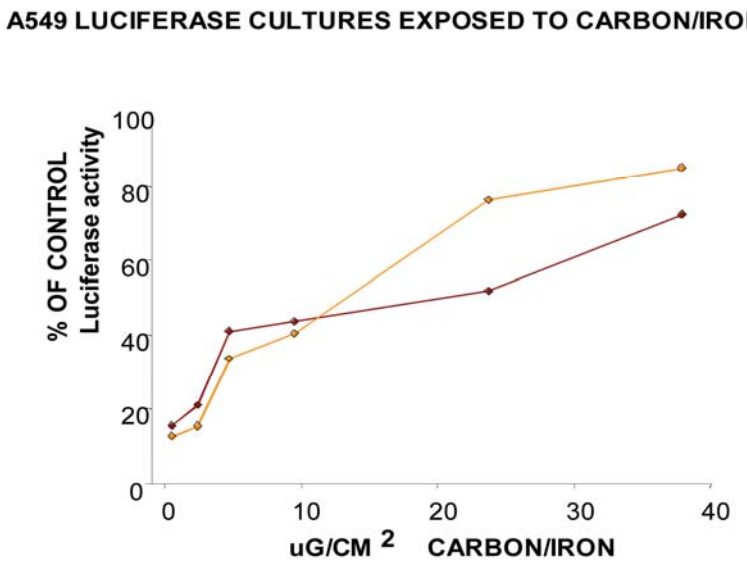

Figure 2. Luciferase Activity in Transfected A549

Particle Effects on Vascular Endothelium

Recent experiments, to better bridge the experiments that are being carried out in the Clinical Studies Core and Animal Exposure Core, have focused on vascular endothelial models that could be useful in assessing particle-induced changes in endothelial gene expression; and that may represent aspects of endothelial dysfunction. To more accurately reflect the complex nature of endothelial interactions with particles, we have used two complementary culture models.

Many of our experiments utilize a standard monolayer culture of primary vascular endothelial cells. A second model, a bilayer epithelial/endothelial co-culture system permits study of cell-cell interactions mediated by particles (Figure 3).

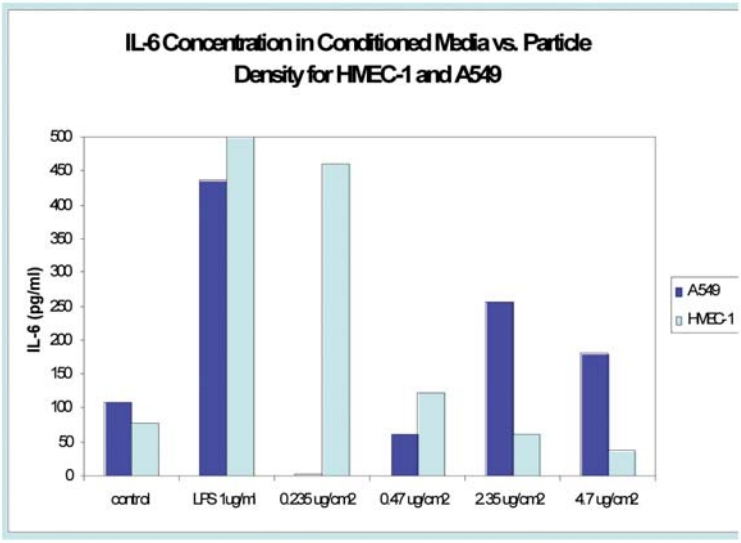

Figure 3. Comparison of IL-6 Release from Epithelial and Endothelial Cells

Monolayer cultures of human umbilical vein endothelial cells (HUVEC) were established and optimized with regard to media, serum, and other culture conditions. Production of IL-6 and PGE_2 when endothelial cells were cultured in the presence of LPS or TNF for 24 hours was used to establish the basic parameters. Our major objective with these cultures was to establish appropriate dose and time parameters of incubation with particles so that we could begin testing with UFP of various compositions. Based on our experiments with cultured epithelial cells, we began our studies using laboratory-generated particles containing 25% Fe. Particles were added to the cells at concentrations ranging from 0.47 to19.0 $\mu g/cm^2$ and media collected at 6 and 24 hours. Particle-induced cytotoxicity was measured by LDH release. Addition of particles in the presence of a priming dose of LPS stimulated the release (production) of both IL-6 and PGE_2 at both 6 and 24 hours. The PGE_2 response appears to be more sensitive as it is observed at doses as low as 0.47 $\mu g/cm^2$ (which converts to a total mass dose of ~1.5 µg of particle) (Figure 4).

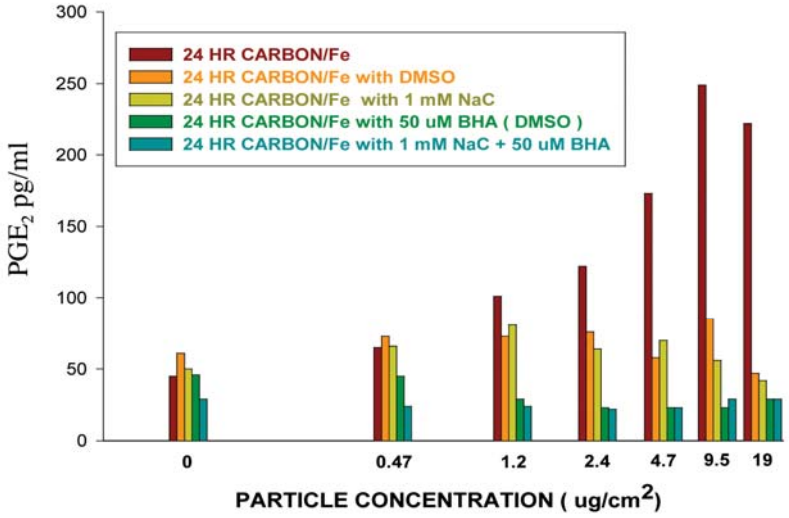

Figure 4. Response of Vascular Endothelial Cells to Particles: Effect of Antioxidants (NAC=N-acetyl cysteine; BHA=butylated hydroxyanisole)

In this system, we also assessed the response of these cells to laboratory-generated carbon, similar to the material used in the human clinical studies, TiO_2, and a laboratory-generated Mn-oxide. In contrast to our experiments with epithelial cells, the endothelial cell cultures were moderately responsive to the carbon alone. After 24 hours of incubation, PGE_2 production was increased 2-3 fold. In contrast C/Fe particles increased PGE_2 by 5-6 fold (Figure 4). Additionally, in support of the role of oxidant stress in particle effects, pretreatment with either a soluble (N-acetyl cysteine) or lipophilic (BHA) antioxidant suppressed the production of PGE_2 induced by PM.

Overall comparison of particle, dose, and time parameters suggest that PGE_2 is the most reliable marker of endothelial activation. It was also noted that particle composition was a major response factor, with TiO_2 being most active and Mn-oxide being most directly cytotoxic. We also determined if coculturing these cells with A549 pulmonary epithelial cells would alter their ability to be stimulated by LPS or by particles. Both cell types appear to be responsive to particles and LPS with apparently different concentration dependence. Using this second model, we have begun to examine cytokine production in response to various stimuli, including particle and LPS. Among the cytokines we evaluated in this model were IL-6 and PGE_2 (Figure 5).

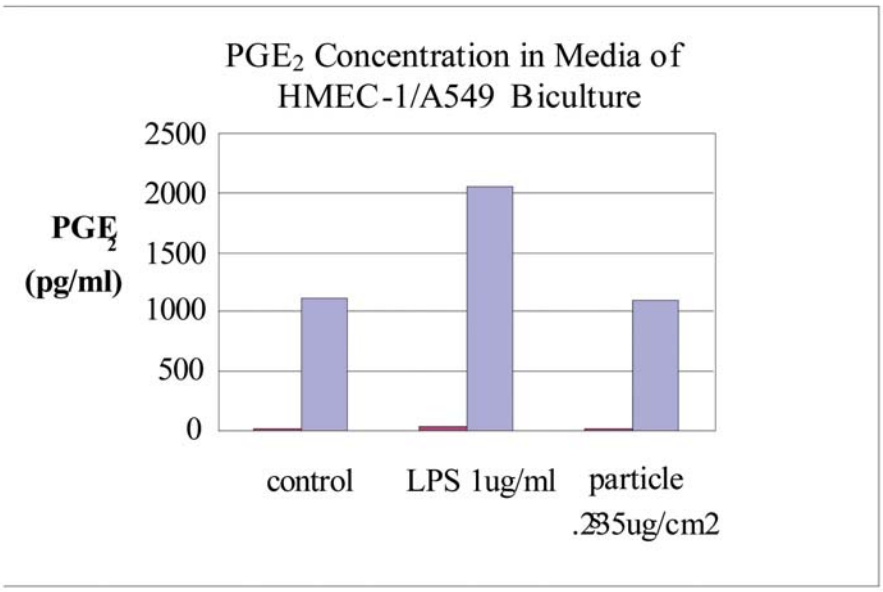

Figure 5. Endothelial Response to Particles: BiCulture Model

The endothelium appears to respond to lower particle mass burdens than does the epithelium. This may account for the enhanced sensitivity of the vascular endothelium *in vivo*. Additionally, aided by our Immunology and Vascular Core, we also measured production of prostaglandins in these culture supernatants. Since the majority of the prostaglandin was found below the membrane, this would suggest it is derived from the endothelium. This is consistent with the *in vivo* results from Core 4 showing enhanced prostaglandin production following particle exposure in a sensitive animal model.

Our experiments have focused on a number of critical issues that relate to the overall goals of the Rochester PM Center. A key point of the studies was the emphasis on real-world particles in lieu of laboratory surrogates. The delay in the characterization of the HUCAPS concentrator in Rochester has delayed this effort somewhat. However, it has enabled us to continue mechanistic studies with defined composition particles that may ultimately be important in attributing effects seen in specific cell populations to unique sources. The one source of real-world particles available for *in vitro* studies was the material collected as part of the MAPS, multi-Center multi-site particle collection effort begun at the end of the grant period.

As shown in Figure 6a and b vascular endothelial cells (HUVEC) exposed to concentrated fine and ultrafine particles respond through increased production of IL-6. This cytokine was chosen as a potential sentinel as a result of experiments from the Animal and Clinical Studies Cores that suggested a possible acute phase response following particle inhalation. Interestingly, using this marker and cell type, we revealed a differential response from particles collected from certain sites. It is hypothesized that this relates to the abundance of vehicle emissions at these sites. More detailed analyses and source calculations are planned with the help of Core 1 as the compositional data are provided.

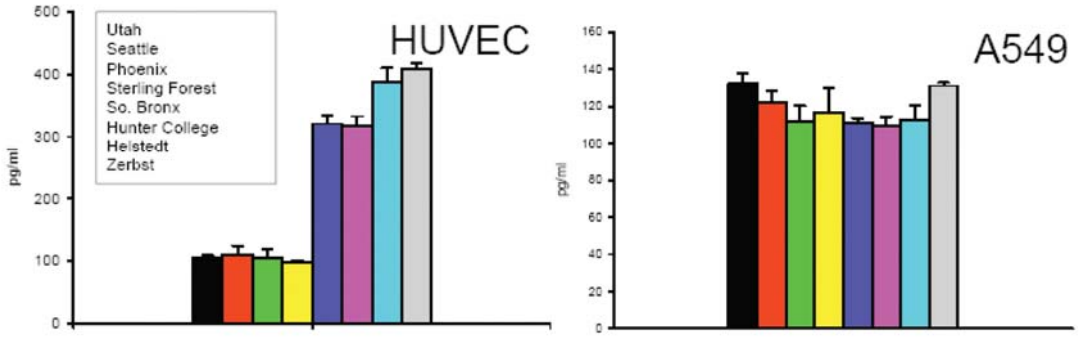

Figure 6. Comparison of IL-6 Production by Cultures Pulmonary Epithelial Cells and Vascular Endothelial Cells Exposed to UFPM Collected From Multiple Sites.

An additional important piece of data was revealed as a consequence of this study. When epithelial cells (A549 cells) were similarly exposed to these materials, no differential response based on site selection was noted. To verify this cell-specific difference and to relate this potential mechanistic difference to studies from Cores 3 and 4, we carried out a direct comparison of the response of the epithelium and vascular endothelium in a series of well characterized particles. We chose these carbon particles as they had previously been used for *in vivo* studies within the Center (Figure 7). While these studies are interesting and important, they do not necessarily address the most relevant question, that of the response of the microvascular endothelium, which is being investigated during the next project cycle.

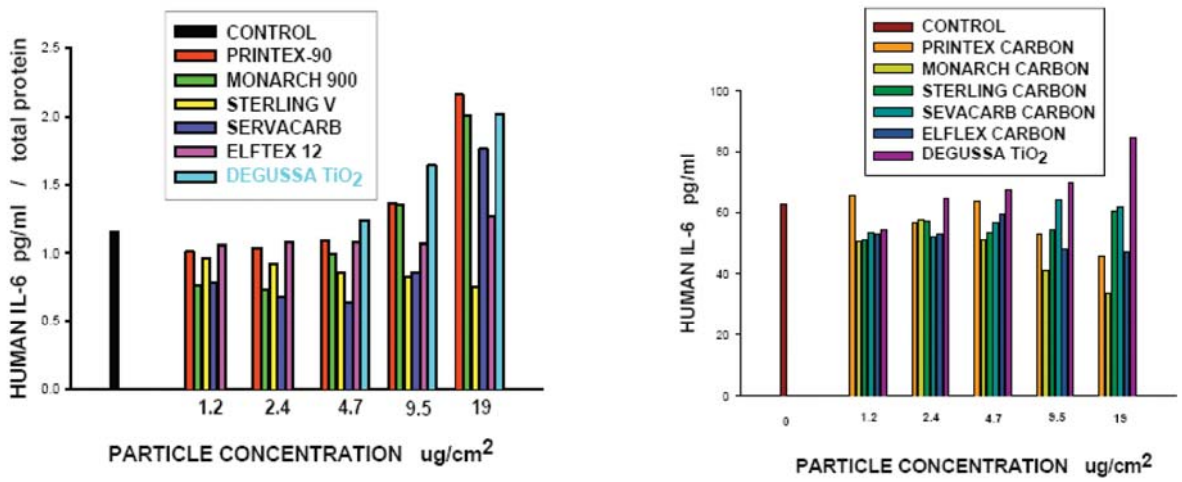

Figure 7. Comparison of IL-6 Production by Vascular Endothelial Cells(a) and Epithelial Cells (b) Exposed to Various Forms of Pure Carbon.

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Supplemental Keywords: NA

Relevant Web Sites: http://www2.envmed.rochester.edu/envmed/PMC/indexPMC.html